Mutational analysis of gap junction formation

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ABSTRACT The paired oocyte cell–cell channel assay was used to investigate the mechanisms involved in the process of formation of gap junction channels. Single oocytes, injected with connexin-specific mRNAs, accumulate a pool of precursors from which cell–cell channels can form rapidly upon pairing. Several lines of evidence, including immunohistochemistry and surface labeling, indicate that part of this precursor pool is located in the cell membrane, probably in the form of closed hemichannels.

The homophilic binding of hemichannels to each other can be mimicked by synthetic peptides representing the extracellular loop sequences of connexin32. The peptides specifically suppress channel formation. A crucial role is established for the six cysteines in the extracellular domains that are conserved in all vertebrate gap junction proteins.

Change of any of these cysteines into serines results in absolute loss of function of the mutant connexin. The effects of thiol-specific reagents on channel formation suggest that docking and/or opening of channels involves disulfide exchange. Several of the variable amino acids in the extracellular loop sequences were found to determine specificity of connexin–connexin interactions.

INTRODUCTION

The cell–cell channels of gap junctions are a unique form of ion channels. They are composed of two hemichannels, each residing in apposing cell membranes, which must line up and interact in order to form a hydrophilic path connecting the cytoplasms of two cells. Each hemichannel is thought to consist of six subunits called connexins. There is a family of connexin proteins that exhibit tissue specificity but very little species (vertebrate) specificity (Beyer et al., 1990).

The formation of a functional cell–cell channel is a fascinating problem because the docking of hemichannels and the subsequent opening of the channel has to be a leakproof process because of the high conductance of these channels. Formally, the docking and opening resembles the processes occurring in ligand-operated ion channels, the difference being that the hemichannel are ligands for each other.

In the past, studies on cell–cell channel formation were limited to the descriptive level. Several of these earlier studies rather than dealing with actual channel formation addressed the process of lateral aggregation of channels that form the gap junction plaques (Abney et al., 1987; Chao et al., 1981; Loewenstein, 1981). Now with sequence information of the protein available and with an efficient functional expression system, experiments become possible to elucidate the molecular mechanisms involved in the channel formation process. The paired oocyte system is particularly suited for such studies because many parameters are under experimental control, in particular timing and macroscopic extent of cell–cell contact are determined by the experimentor.

Here we identify determinants of cell–cell channel formation that are not part of the channel proteins themselves (extrinsic determinants) as well as domains within the connexin protein (intrinsic determinants). The critical role for the conserved cysteine residues in the extracellular loops is being established. Apparently variable amino acids in the otherwise highly conserved extracellular domains of the connexins seem to provide specificity for the interaction between different connexins.

METHODS

Preparation of oocytes

Xenopus laevis oocytes were isolated from the ovaries by collagenase treatment. Oocytes were injected with ~50 nl of connexin mRNA. 18 to 24 h later, the vitelline layer was removed with forceps. These stripped oocytes were then incubated for 20 min with 10 μg/ml lectins (soybean agglutinin if not stated otherwise). Subsequently, oocytes were washed and paired with the vegetal poles facing each other (Levine et al., 1988).

Measurement of junctional conductance

Junctional conductance between oocytes was determined 2 h (unless stated otherwise) after pairing with the dual voltage clamp technique (Spray et al., 1981). All experiments included uninjected oocyte pairs as negative controls as well as oocytes injected with wild-type connexin32 as positive controls. When data from different oocyte prepara-

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tions were compared, junctional conductances were normalized to the positive controls.

Source of connexin mRNA

Connexin32 mRNA was transcribed in vitro from a cDNA clone (Paul, 1986), that was modified at the 5′-end and subcloned in pGEM-3Z as described (Werner et al., 1991). Connexin43 mRNA was prepared similarly from a cDNA clone (Beyer et al., 1987) modified at the 5′-end as described (Werner et al., 1989). Connexin38 mRNA was prepared from a cDNA clone isolated from a *Xenopus* oocyte cDNA library (W. Yu, M. Chang, and R. Werner, unpublished data). As the other cDNA clones, this clone was modified at its 5′-end to introduce the same Kozak (1986) consensus sequence that was introduced into the other cDNA clones in order to warrant similar translational efficiencies. Mutagenesis was done as described previously (Dahl et al., 1991).

Immunohistochemistry

An antibody against the cytoplasmic C2 domain of connexin32 (amino acids 104–122) was generated with a synthetic peptide injected into rabbits. A detailed description of the immunohistochemical techniques and of other aspects of the oocyte cell–cell channel assay is given elsewhere (Dahl, 1991).

RESULTS AND DISCUSSION

Rapid channel formation from a pool of precursors

When oocytes are injected with connexin-specific mRNA and are incubated as single oocytes for several hours, they accumulate a pool of precursors (Dahl et al., 1988, 1991; Werner et al., 1989). Cell–cell channels can form rapidly from this pool upon pairing of the oocytes (Fig. 1). Assuming a unit conductance of ~100 pS for the connexin32 channel, the initial channel formation rate can be in excess of 40 channels opening per second. During the period of rapid channel formation no change of the nonjunctional membrane resistance is detectable. If the mRNA is injected into oocyte pairs, junctional conductance becomes only detectable after a delay of 4–6 h and then gradually increases (Dahl et al., 1988). With increasing time periods between mRNA injections and pairing, the channels become detectable earlier and the initial rate of channel formation increases. This suggests that the precursor pool grows with time and consequently synthesis of channel proteins must exceed their degradation.

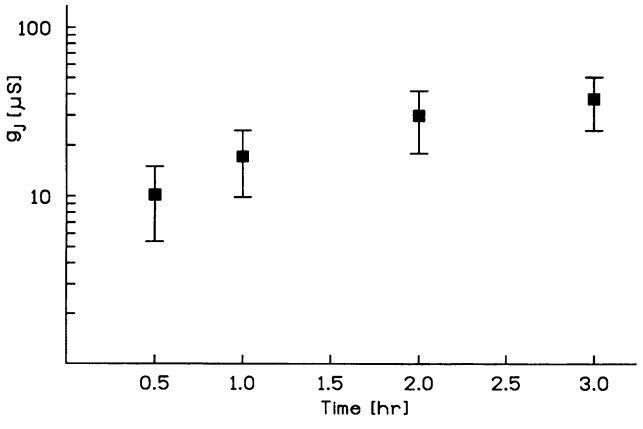

FIGURE 1 Time course of cell–cell channel formation. Junctional conductance (g_j) is expressed in μS, and the means ± SEM (n = 9) are plotted as a function of time after pairing at time point zero. Oocytes were reacted with 10 μg/ml of soybean agglutinin for 20 min, washed, and then paired. Connexin32 mRNA had been injected 24 h earlier.

Where is the pool of precursor located?

In organized tissues and cultured cells, immunohistochemical staining of gap junction proteins shows the stain to be concentrated in areas where gap junction plaques are located, giving rise to a typical punctate staining pattern (Paul, 1986). This means that there is no sizeable pool of intramembraneous gap junction precursors or that the antibodies used selectively recognize only the proteins aggregated into gap junction plaques.

In *Xenopus* oocytes overexpressing connexins, however, a series of observations indicates that at least a fraction of the pool is localized in the membrane. First, anticonnexin32 antibodies show that there is cytoplasmic staining, but in addition there is a strong signal that can be localized in, or very close to, the plasma membrane (Fig. 2 *a*). In fact, the staining pattern seen associated with the membrane is indistinguishable from that seen when an authentic membrane protein is labeled from outside as shown for the agglutinin receptor (Fig. 2 *b*).

Additional evidence for membrane localization of gap junction precursors comes from surface labeling experiments. Connexin mRNA-injected oocytes have a larger number of thiol groups exposed on their extracellular surface than uninjected oocytes (Dahl et al., 1991). This is accounted for by the presence of six cysteine residues in the extracellular domain of each connexin subunit. In addition, reaction with thiolspecific reagents has been shown to deplete the cell–cell channel precursor pool.

In a similar fashion this pool can be depleted by treatment of the oocyte surface with low concentrations of trypsin. The time course of channel formation observed after trypsinization resembles that of oocytes that were paired immediately after mRNA injection and therefore have no precursor pool (Dahl et al., 1991).

If the precursor were not located in the plasma membrane one would have to invoke a mechanism whereby membrane contact provides a signal for membrane insertion of precursors. In fact, the proposal for a

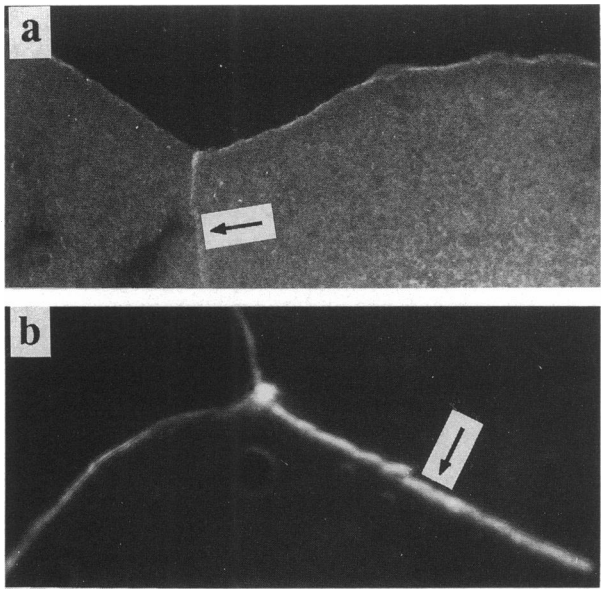

FIGURE 2 Immunohistochemical localization of connexin32 (*a*), surface labeling of oocytes with fluorescent wheat germ agglutinin (*b*). Connexin32 is present in the cytoplasm as well as in a compartment that is close to or in the plasma membrane. The same membrane staining pattern, although at higher intensity, is seen for the agglutinin receptor. The relatively broad staining pattern of the membrane is due to the microvillar surface of the oocyte. No apparent difference in the staining intensity is seen between the free membrane surface and the interface (*arrows;* note that at the interface the signals add up). The antibody was prepared against a peptide representing the amino acid sequence 104–122 of connexin32. Antibody-staining was performed on cryosections; the agglutinin was applied to intact oocytes.

shift of gap junction proteins from a cytoplasmic compartment into contacting membranes has been made (Swenson et al., 1989; Musil et al., 1990). However, tests of this hypothesis did not support the existence of such a signaling mechanism. Conditioning contacts between cells had no effect on the rate of channel formation (Levine et al., 1991).

Taken together, these different lines of evidence strongly support the existence of a channel precursor pool in the plasma membrane.

Is the precursor in monomeric or oligomeric form?

Most multimeric membrane proteins studied so far have been shown to be assembled on intracellular membranes. This includes membrane proteins as diverse as the nicotinic acetylcholine receptors and Influenza virus hemagglutinins. It is even thought that multimerization is a prerequisite for the exit of such proteins from the endoplasmic reticulum (Paulson et al., 1991; Ceriotti and Coleman, 1990).

In analogy, therefore, one would expect the cell–cell channel precursor to exist in a similar multimeric form when present in the plasma membrane. When the membrane resistance is measured in oocytes expressing a large pool of channel precursors, no evidence for open hemichannels is seen. Thus, if hexameric hemichannels exist, they must be in a closed state (Werner et al., 1989).

Indirect evidence for the existence of a membrane pool of hemichannels comes from measurements of the rate of channel formation as a function of mRNA concentration (Fig. 3). In a double logarithmic plot of the number of open channels formed (junctional conductance) versus concentration of mRNA (proportional to protein level), a slope of two is observed when the concentration is changed in both oocytes of a pair. If, on the other hand, one oocyte receives a saturating concentration of mRNA, whereas the other receives various dilutions, a slope of one is observed. This shows that oligomerization of subunits cannot be a rate-limiting step. Cell–cell channel formation thus exhibits the properties of a bimolecular reaction. However, firm conclusions on the assembly states of the precursor molecules must await thorough analysis by sedimentation studies or change of electrophoretic mobility in response to cross-linking.

Extrinsic determinants of channel formation

The fast rate of channel formation shown in Fig. 1 is usually not seen unless the oocytes are doped with lectins. Without lectins there is considerable variability

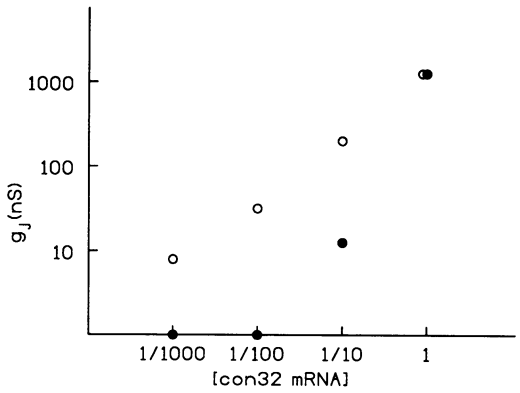

FIGURE 3 Junctional conductance as a function of the concentration of connexin32 mRNA that is injected. Oocytes were prepared as described in Fig. 1, except that junctional conductance was determined 2 h after pairing. Each point represents the mean of nine oocyte pairs. Solid circles represent pairs where both oocytes were injected with various mRNA concentrations. Open circles represent pairs where one oocyte received the highest mRNA concentration and the other various dilutions of mRNA.

in the level of conductances in oocyte pairs from different donors. Lectin treatment tends to reduce this variability and, in addition, ~10× higher conductances are observed. It is tempting to assume that the lectins exert their effect on junction formation by their adhesive properties. This appears to be in line with a proposal that adhesion molecules are essential for gap junction formation (Keane et al., 1988; Mege et al., 1988; Musil et al., 1990). A systematic study of the lectin effect on cell–cell channel formation in paired oocytes, however, suggests that adhesion can only be a minor contributing factor, if any (Levine et al., 1991).

Several lines of evidence including lectin binding studies, time course of the effect, electron microscopy, indicate that the lectin effect occurs mainly on the basis of removal of steric hindrance by clustering of the lectin receptors and by endocytosis. A key experiment involves lectin-specific sugars. Such sugars inhibit the lectin effect on channel formation only if they are added before the lectins have bound to their receptors. If the sugars are added later, the lectin effect on channel formation remains unchanged. This is observed with a large excess of sugars over lectins where all free valancies are occupied, thus preventing the agglutination reaction mediated by lectins. Consequently, the effect of the lectins is exerted on the single oocyte.

Additional evidence for the clearing hypothesis (removal of steric hindrance) comes from the following observations. The lectin effect is unspecific, and the effects of different lectins are additive. The combined effect of lectins that bind to different sugars, such as concanavalin A and wheat germ agglutinin, is larger than the effect of each individual lectin applied at twice the concentration. Furthermore, removal of sugar moieties from the oocyte surface with *N*-glycosidase F also increases the rate of cell–cell channel formation (Fig. 4).

Considering the large dimensions of glycoproteins, which includes adhesion molecules, the direct involvement of adhesion molecules in channel formation is not appealing. From what is presently known about the structure of cell–cell channels, each hemichannel does not protrude more than 10 Å from the membrane surface. Thus, a glycoprotein is likely to provide a formidable hindrance to the interaction of two hemichannels. The observed effects of adhesion molecules in other systems on intercellular communication (Keane et al., 1988; Mege et al., 1988; Musil et al., 1990), therefore, may be attributable to other steps rather than channel formation per se. For example, membrane encroachment in the oocyte system is done by the experimenter. In cultured cells, on the other hand, the cells have to do it on their own. From published figures it appears that adhesion molecules are instrumental in this step as indicated by the gross morphological changes that, for example, occur after expression of adhesion molecules by transfection or after blocking neuronal cell adhesion molecules (N-Cam) with antibody.

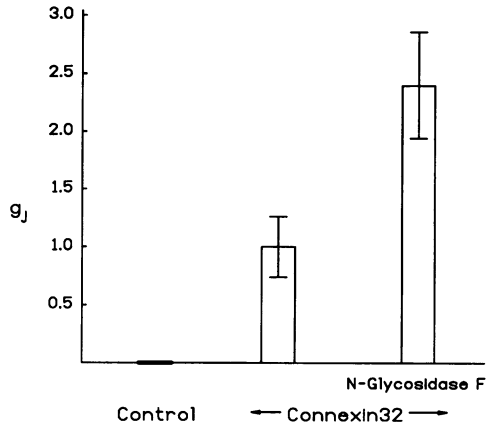

FIGURE 4 Effect of treatment of the oocyte surface with *N*-glycosidase F on the formation of cell–cell channels. Junctional conductance was determined 2 h after pairing. Negative control (uninjected oocytes) and positive control (connexin32 mRNA-injected oocytes) remained untreated. In the experimental group, oocytes were treated with 2 units/ml of *N*-glycosidase F for 30 min at pH 7.5, washed, and then paired. Means ± SEM (n = 9). All data were normalized to the positive control. No lectins were used in this experiment.

Some adhesion molecules, such as the cadherins, require Ca^{2+} ions for activity. We tested the effect of extracellular Ca^{2+} concentration upon the rate of channel formation. Surprisingly, the physiological extracellular concentration of Ca^{2+} ions is not optimal for channel formation. Reducing extracellular Ca^{2+} concentration increases the number of open channels formed up to a plateau that is reached at 0.1 mM (Fig. 5). Reduction of extracellular Mg^{2+} concentration had a similar effect.

It is unusual that the physiological concentration of ions is not optimal for a biological process occurring in that environment. Perhaps the prevaling Ca^{2+} concentration in areas where junctions form is lower. Such microenvironments conceivably could exist in the narrow clefts where junctions usually form in organized tissue, in particular, in the vicinity of tight junctional seals. It is an old observation that gap junctions are intimately associated with tight junctions in cells, as epithelia, that express both.

Alternatively, cell–cell channel formation may be completely independent of extracellular divalent cations. The observed effect may then need to be attributed to other divalent cation-dependent processes that may interfere unspecifically with channel formation rate. Thus, adherins have to be considered candidates for the calcium effect.

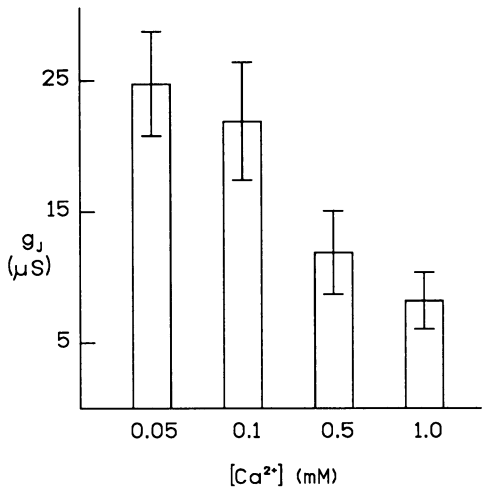

FIGURE 5 Junctional conductance as a function of extracellular calcium concentration. Oocytes were prepared as described in Fig. 1. They were paired and incubated in the presence of various Ca^{2+} concentrations. Conductance was determined 2 h after pairing.

Intrinsic determinants of channel formation

The connexins presently are among the best-mapped proteins with multiple membrane-spanning domains. Extensive studies in different laboratories (Hertzberg et al., 1988; Milks et al., 1988; Goodenough et al., 1988) using site-specific antibodies and limited proteolysis have established the membrane topography of this protein as shown in Fig. 6. There are two extracellular loops, four transmembrane segments, and three cytoplasmic domains including both amino- and carboxy-termini. Whereas this general structure is well documented, the boundaries at the membrane surfaces are arbitrarily set because exact measurements are still lacking. One also has to consider the possibility that the boundaries change for the different physiological states, e.g., closed hemichannel versus open channel.

Cell–cell channels can be, but do not need to be, made of one single type of protein. The interaction of hemichannels, therefore, is homophilic. Thus, one would

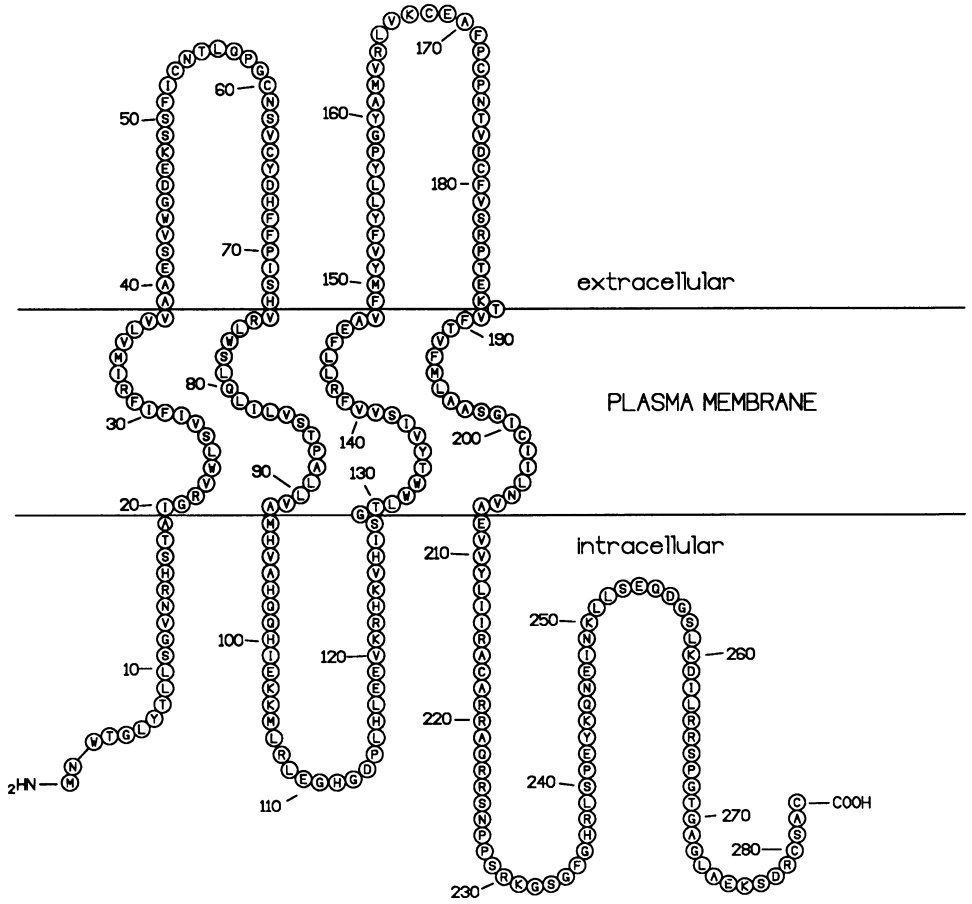

FIGURE 6 Transmembrane topography of connexin32.

predict that the addition of a peptide with the sequence of the extracellular segments should bind to the loops. If the binding of such peptides precedes the pairing of oocytes, cell–cell channel formation should be impaired, provided that the peptides assume the proper configuration in solution and binding is sufficiently tight so as to disallow displacement by intact subunits.

Such inhibition of channel formation by peptides is indeed observed. Synthetic peptides representing the amino acid sequences of either of the two extracellular loops (aa39–aa76, aa164–aa189) of connexin32 inhibit channel formation. The effect appears to be specific, the cysteine-containing peptide oxytocin as well as a peptide representing the intracellular C2-segment of connexin32 did not affect channel formation (Fig. 7).

The peptide concentration required (10^{-4} M) appears to be high. However, in a similar approach to the study of binding of G protein to rhodopsin (Hamm et al., 1988) equally high concentrations of peptides were needed. It is conceivable that only a fraction of the peptide is in the conformation permissive for binding. Alternatively, the entire population due to conformational constraints may exhibit low affinity. More detailed mapping of the binding sites with shorter peptides is in progress.

Although inhibition of channel formation clearly occurred, the binding of the peptide to the extracellular loops of hemichannels must be different from the binding that occurs between two hemichannels. Such a binding results in the opening of the two interacting hemichannels, whereas the peptide used in this experiment did not open the hemichannel in a detectable way.

The extracellular domains of all known vertebrate connexins contain three cysteine residues located at

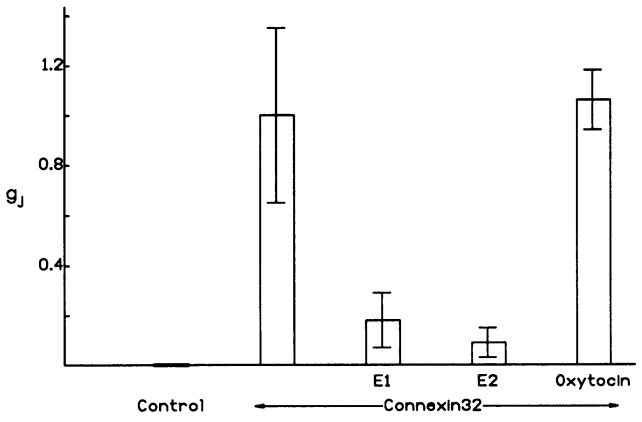

FIGURE 7 Effect of peptides, representing extracellular loop sequences E1 and E2 of connexin32, on cell–cell channel formation. The two synthetic peptides and oxytocin were applied at concentrations of 10^{-4} M to the oocytes 10 min before pairing. Junctional conductance was measured 2 h after pairing. mRNA was injected 24 h before pairing. Data were normalized to the positive control. Means ± SEM (n = 9).

identical positions (with the exception of connexin31, where one cysteine is shifted by one position). Loop 1 contains the motif CX_6CX_3C, loop 2 the motif CX_4CX_5C. This high conservation of cysteine residues suggests an important structural function. Change of any one of these cysteine residues into a serine residue by site-directed mutagenesis resulted in absolute loss of function (Fig. 8; Dahl et al., 1991). This is true for symmetric assays with both oocytes in a pair expressing the mutant protein, as well as for the less stringent asymmetric assay where one oocyte expressed wild-type hemichannels.

Six other mutations in the extracellular loops involving amino acids in the vicinity of the cysteines all affected channel formation ability. However, none of the mutations resulted in the complete loss of function as was observed with the six cysteine mutants. They all showed activity, at least when tested in the asymmetric assay against wild-type connexin32 (Fig. 8).

In contrast, a mutant in which cysteine-217 was changed to serine, located within the cytoplasmic domain C3, as well as a deletion mutant (Werner et al., 1991) that lacked the carboxyl terminus including two additional cysteines, exhibited the same channel-forming capabilities as wild-type connexin32 (Fig. 8).

These data show that the extracellular cysteines provide a crucially important function in any one of the stages involved in channel formation from early assembly to final channel opening. Because the electrophysiological assay relies solely on open channels, no conclusion can be drawn where the cysteine mutants fail. As outlined earlier, the exit of multimeric membrane proteins requires assembly in the endoplasmic reticulum or Golgi. If the assembly is interfered with, then the mutant protein should not be found in compartments beyond the Golgi. Immunohistochemical analysis, however, reveals that each of the six cysteine mutants has the same distribution as wild-type connexin32 including the plasma membrane compartment. This suggests that for the mutants tested assembly is at least partially possible. This leaves two processes that may require functional cysteines: the docking (binding) of hemichannels and/or channel opening.

Consistent with the properties of the cysteine mutants is the effect of thiolspecific reagents upon the formation of open channels. The membrane-impermeant compound maleimidobutyrylbiocitin (MBB) inhibits channel formation when applied to the outside of the oocyte but only after reduction of disulfide groups. The same reduction is required for the binding of MBB to the oocyte surface as determined by avidin binding to the biotin moiety of MBB (Dahl et al., 1991). This suggests that the extracellular cysteines are oxidized.

The effect of reducing agents such as mercaptoethanol or dithiothreitol is paradoxical. At high concentra-

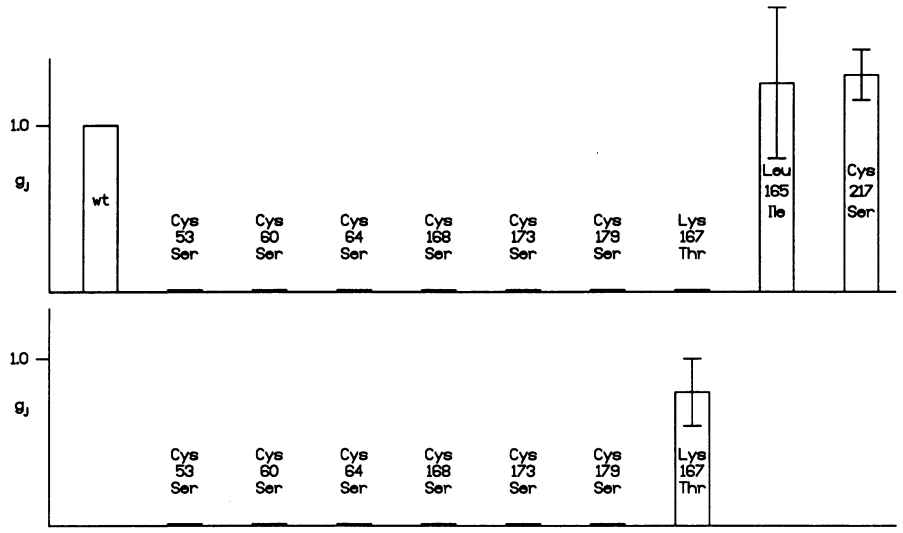

FIGURE 8 Effects of single amino acid changes in the extracellular domains on channel formation. mRNA was injected 24 h before pairing and junctional conductance was measured 2 h after pairing. Equal concentrations of mRNA, as tested by agarose gel electrophoresis, were injected in each oocyte. Conductances were normalized to wild-type connexin32. (*a*) Symmetric pairs: both oocytes were injected with the same mRNA. (*b*) Asymmetric pairs: one oocyte received wild-type connexin32 mRNA, the other mutant connexin32 mRNA. Means ± SEM (n = 9).

tions they inhibit channel formation, at intermediate concentrations they are ineffective, and a low concentrations they stimulate channel formation. This paradox can be explained by disulfide exchange reactions that are involved in the channel formation process. This is supported by the observation that high extracellular pH stimulates channel formation and that this effect is additive to that of low concentrations of reducing agents (Dahl et al., 1991).

Based on these observations the following hypothesis emerges. Channel precursors are or become rapidly oxidized when they are inserted in the plasma membrane. For docking and/or for the opening of channels disulfide exchange reactions are required. The disulfide bridges that exist in hemichannels and those that form during channel formation are not localized at this time. They could be either intramolecular, intermolecular

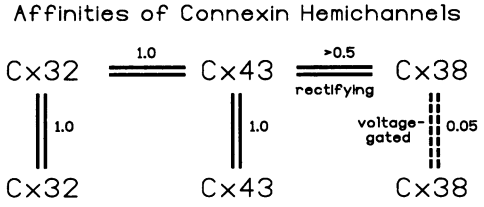

FIGURE 9 Relative affinities of connexins to each other. The indicated numbers represent normalized (connexin32 versus connexin32) conductances measured 2 h after pairing. Equal concentrations of mRNA were injected 24 h before pairing.

within a hemichannel, or intermolecular between hemichannels.

Specificity of hemichannel interaction

Earlier studies had shown that mRNA-injected oocytes can recruit endogenous channel activity in noninjected oocytes that was not detectable when two noninjected oocytes were paired (Werner et al., 1985). This combination of a hemichannel from a voltage-independent junction (uterine or heart mRNA) with a hemichannel of a symmetrically voltage-dependent junction (endogenous) resulted in a rectifying channel (Dahl and Werner, 1986; and Dahl and Werner, unpublished data). These findings were subsequently confirmed by the discovery that hemichannels of connexin43 form hybrid channels with endogenous oocyte hemichannels that exhibit the same rectifying properties (Werner et al., 1989; Swenson et al., 1989). The endogenous channel activity appears to be due to connexin38, the oocyte-specific connexin (Ebihara et al., 1989).

The perplexing observation was made that the number of hybrid channels formed in different oocyte preparations between connexin43 and endogenous hemichannels varied to a large extent, whereas corresponding purely endogenous activity was either nonexistent or low. This observation makes it unlikely that recruitment of endogenous hemichannels on the basis of mass action could be the only cause for the appearance of high levels

of hybrid channels. Therefore, the channel-forming ability of connexin38 was tested directly against that of connexin43 and connexin32.

Surprisingly, the same levels of mRNA resulted in similar levels of junctional conductance for connexin43 or connexin32, whereas the channels formed from connexin38 yielded a conductance that was 20 times lower. On the other hand, connexin38-connexin43 hybrid oocyte pairs exhibited ~50% of the conductance observed in pairs symmetrically expressing connexin43. This excludes the possibility of the connexin38 single channel conductance being considerably lower than that of the other types of channels. Note that here and for the interpretation of subsequent data the assumption is made that docking specificity does not drastically affect the conductance properties of the hemichannels involved. This assumption appears to be justified considering the different rates of formation of junctional conductance achieved with different connexins and their mutants (Werner et al., 1992). No hybrid channels formed between connexin38 and connexin32, a result consistent with the observation that connexin32 did not form hybrid channels with endogenous channels (Swenson et al., 1989).

When given the choice connexin43 hemichannels will rather pair with connexin43 hemichannels than with connexin 38 hemichannels in the other oocyte. This is indicated by the low level of voltage dependence of symmetrical oocyte pairs that were injected with mixtures of connexin43 and connexin38 mRNA. Similarly, in such pairs low CO_2 concentrations, which selectively knock out connexin38 channels both in the hybrid and the symmetric form (Werner et al., 1991), lead only to a marginal reduction in the macroscopic conductance. From this one has to conclude that the affinity between different gap junction proteins varies (Fig. 9). It appears that connexin38 may be designed to form hybrid channels rather than channels with itself. The preference is for connexin43 rather than connexin32. However, changing specific amino acids in the extracellular loops of connexin32 into amino acids found at the same positions in connexin43 can change the affinity sufficiently so that mutant connexin32–connexin38 hybrid channels form. For example, connexin32 with I-52 and K-167 replaced by R and T, respectively, forms a rectifying channel with connexin38 (to be published elsewhere). Thus, it appears that the conserved segments of the extracellular loops play a critical role in the basic docking and opening process whereas the apparently variant segments confer specificity to the docking process.

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DISCUSSION

Session Chairman: Alan Finkelstein *Scribes:* Yinong Zhang and Juan Pascual

DAVID SPRAY: If the hemichannel exists in a plasma membrane, and if it opens, even briefly, the cell must die. So the channel must stay in the closed state. Immunofluorescence has never identified populations of immunoreactive materials on the surface of any wild-type cell.

GERHARD DAHL: I agree with the first statement that a hemichannel has to be closed. We find no change of input resistance between uninjected oocytes and oocytes overexpressing connexins. The following observations suggest that precursors are in the membrane. This evidence is for precursors that are not necessarily hemichannels.

First, if we look at immunohistochemical staining of oocytes we find intense staining in the vicinity of, if not within, the plasma membrane. This staining pattern can not be distinguished from that obtained by labeling an authentic membrane protein from the outside such as the agglutinin receptor.

Second, the staining pattern with connexin antibody is the same for single oocytes and paired ones. In pairs, the staining is continuous from the junction-containing interface to the free surface.

Third, we can indirectly demonstrate that connexins are accessible to surface labeling: when oocytes are first reduced and then reacted with maleimide-butyryl-biotin, a significantly higher amount of avidin binds to oocytes overexpressing connexin-32 than to control oocytes.

Finally, just recently Musil and Goodenough published a paper (1991. *J. Cell. Biol.* 115:1357–1374) showing that the connexin protein proper can be biotinylated from the outside. In reference to immunoreactive material on the surface of wild-type cells, Rahman and Evans show in a recent publication (1991. *J. Cell. Sci.* 100:567–578) that a faint labeling at the level of the plasma membrane can be seen in hepatocytes with antibodies directed against extracellular loop sequences of connexin-32.

RICHARD HORN: Is it possible for you to pull the paired oocytes apart after the functional channel has been formed and to study the conductance of the hemichannels?

DAHL: Yes, you can pull the cells apart. However, the result will be that one or both oocytes will die. What happens is that junctions stay with one cell and are torn out of the other cell together with a patch of nonjunctional membrane. The defects created this way often do not seal over. This not only happens in oocytes but also in almost all cells during tissue dissociation into single cells. The binding between hemichannels is so tight that you need 8 M urea to separate isolated gap junctions into hemichannels.

ANDREW HARRIS: Those experiments with 8 M urea were done on junctions isolated under harsh conditions that select for insoluble structures and yield a small percentage of the junctions in the tissue. Hyperosmotic treatment of a tissue can split junctions, but this is probably due to a cellular reponse to the osmotic change, not a direct effect on the junctions. We really don't know what holds the gap junction together.

B. VEERAPANDIAN: Do you think that these three cysteines form disulfide bonds with other ones? Do they form intra- or inter-disulfide bonds?

DAHL: I think we should make a clear distinction between connexins in a fully assembled channel in a gap junction, and connexins in channel precursors. Both may even need to be distinguished from connexins during the channel formation process. Nothing is known about the disulfides in the precursors. Revel's (John and Revel. 1991. *Biochem. Biophys. Res. Commun.* 178:1312–1318) group and Evans independently have recently proposed that in organized gap junctions disulfide bonds are exclusively intramolecular and that at least one disulfide bond connects the two extracellular loops. The evidence is that after cutting connexins with protease between the two loops, two peptides are detectable only after reduction. I would like to reemphasize that different bonds may exist at different times during channel formation, which could include transient intermolecular disulfide bonds. In fact, we have published data suggesting the possibility of disulfide exchange reactions in connexin-connexin interactions. This includes the catalytic effect of low concentrations of reducing agents and the pH effect on channel formation.

ROGER KOEPPE II: Have you tested whether any of the nonfunctional cysteine mutants can "complement" each other, by injecting mRNAs representing two different Cys mutations into the same oocyte, in either a symmetric or asymmetric assay of the type shown in Fig. 8? The simplest way to do this would be to add wild-type connexin mRNAs to one oocyte and all six Cys mutant mRNAs to the other oocyte. If any channels were observed, you could then omit one or more of the mutant mRNAs in future experiments.